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(71) Applicant *(for all designated States except US)*: PHARMALINK INTERNATIONAL LIMITED [-/CN]; Sixth Floor Terrace, 8 Duddell Street, Central, Hong Kong (CN).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: MACRIDES, Theodore [AU/AU]; 13 Exeter Close, Lower Templestowe, VIC 3107 (AU). KALAFATIS, Nicolette [AU/AU]; 51 Chatsworth Road, East Prahran, VIC 3181 (AU). BETTS, Henry, W. [AU/AU]; The Queen Elizabeth Hospital Rheumatology Research Laboratories, Clinical Development Research Centre, 28 Woodville Road, Woodville South, S.A. 5011 (AU).

(74) Agents: SLATTERY, John, M. et al.; Davies Collison Cave, 1 Little Collins Street, Melbourne, VIC 3000 (AU).

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(54) Title: INHIBITOR OF LIPOXYGENASE PATHWAYS

(57) Abstract

A method of inhibition of a lipoxygenase pathway, particularly for the treatment of a disease or condition associated with a lipoxygenase pathway, in a human or animal patient which comprises administration to the patient of an effective amount of a lipid extract of *Perna canaliculus* or *Mytulis edulis*.

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INHIBITOR OF LIPOXYGENASE PATHWAYS

FIELD OF THE INVENTION

This invention relates in general to a preparation having effective activity as an inhibitor of the lipoxygenase pathways, the preparation being a lipid extract of mussels, including the New Zealand green-lipped mussel, *Perna canaliculus*, and the blue mussel, *Mytilus edulis*. In particular, the invention relates to the use of this preparation as a prophylactic or therapeutic agent in inhibition of lipoxygenase pathways, particularly the 5- and/or 12-lipoxygenase pathways, for example in the treatment of cancer by inhibiting tumour cell proliferation and tumour metastasis, as well as in the treatment of asthma, atherosclerosis and other diseases or conditions associated with a lipoxygenase pathway.

BACKGROUND OF THE INVENTION

The role of the metabolites of arachidonic acid in inflammation has been well established, and many of the current therapeutic agents for the treatment of inflammation involve the inhibition of arachidonic acid metabolism. For example, many of the non-steroidal anti-inflammatory drugs inhibit the formation of prostaglandins and thromboxane via the cyclo-oxygenase pathway.

The metabolism of arachidonic acid via the 5- lipoxygenase pathway of leukocytes leads to the formation of leukotriene B_4 (LTB_4) and the leukotrienes C_4, D_4 and E_4 (LTC_4, LTD_4 and LTE_4) as shown in Figure 1. LTB_4 is a potent chemotactic agent and is responsible for the increased number of leukocytes at sites of inflammation. LTC_4, LTD_4 and LTE_4 are very potent broncho-constricting agents produced by eosinophils in the lung, and whose production is massively increased during an asthma attack.

Zileuton (Zyflo™, Abbott) is a selective, orally active inhibitor of 5-lipoxygenase, and this product has been shown to exert anti-inflammatory and anti-allergic effects in animal models and humans. It is used for the prevention and chronic treatment of asthma in patients of at least 12 years of age.

As shown in Figure 1, another major metabolite of the 5-lipoxygenase pathway is 5-hydroxyeicosatetraenoic acid (5-HETE). Until recently there were no known major physiological roles for 5-HETE. Recent research has, however, demonstrated that 5-HETE is involved in the proliferative response of cancer cells. In addition, it has also been demonstrated that the product of the 12-lipoxygenase pathway, 12-HETE, is involved in tumour metastasis.

The role of lipoxygenase metabolites such as 5-HETE and 12-HETE in cancer is demonstrated by the following observations:

(i) Increased production of 5-HETE and 12-HETE in tumour cells

In the human prostate cancer cells, 12 -lipoxygenase mRNA levels are elevated compared to normal cells, such expression correlated with poor differentiation and invasiveness of the tumour (Gao *et al.*, 1995).

In patients with breast cancer, the levels of 12-lipoxygenase mRNA were higher compared to normal breast tissue, and similar findings were observed in cultured breast cancer cells compared to normal epithelial cells (Natarajan *et al.* 1997).

Cultured human prostate cancer cells (PC-3) fed with arachidonic acid showed an increased production of 5-HETE, which was blocked by the 5-lipoxygenase inhibitor MK886 (Ghosh and Myers, 1997).

Mice treated with urethane to induce lung tumours had higher levels of PGE_2 and HETEs in the lung tissue compared to control mice (Ichikawa *et al.*, 1997).

(ii) 5-HETE stimulates tumour cell proliferation.

In the human prostate cancer cell line (PC-3) cultured with arachidonic acid the increased cell growth correlated with the amount of 5-HETE synthesised (Ghosh and Myers, 1997). The 5-lipoxygenase inhibitor MK886, but not 12-lipoxygenase inhibitors, inhibited this effect, which was reversed by the addition of exogenous 5-HETE (Ghosh and Myers, 1997).

In cultured human breast cancer cells (HS578T), the lipoxygenase inhibitors nordihydroguairetic acid and esculetin, but not the cyclo-oxygenase inhibitor piroxicam, suppressed cell growth (Hofmanova *et al.*, 1996).

In patients with breast cancer, the 5-lipoxygenase inhibitor, tamoxifen, has been clinically effective in prolonging life (Tavares *et al.*, 1987).

Both in *in vivo* and *in vitro* studies of colon cancer in mice using MAC26 and MAC13 tumour cells, low concentrations of linoleic acid and arachidonic acid stimulated cell growth. This growth was inhibited by cyclo-oxygenase and lipoxygenase inhibitors indomethacin and BWA4C (Hussey and Tisdale, 1996).

In the human pancreatic cell line (Panc-1), the 5-lipoxygenase inhibitor MK886 induced cell death (Anderson *et al.*, 1998).

In mice with Lewis lung cancers, cancer cell growth and metastasis was inhibited following administration i.p. of minocycline and phenidone-

cyclo-oxygenase and lipoxygenase inhibitors respectively (Teicher *et al.*, 1994).

(iii) 12-HETE promotes tumour metastasis

Tumour metastasis is characterised by a variety of quite distinct physiological processes including detachment of tumour cells from the primary tumour, intravasation from the primary tumour site into the blood stream, adherence to blood vessel endothelium at a remote sight, induction of endothelial cell retraction and extravasation and migration to a new tissue site. Interactions between tumour cells and platelets which produce 12-HETE are very important in the process of retraction and extravasation (Honn *et al.*, 1994a)

Cultured amelanotic melanoma cells (B16a) are found in two forms. The high metastatic cells (HM340) generated high levels of 12-HETE and low levels of 5-HETE, whereas the low metastatic line (HL180) generated only low amounts of both HETEs. The lipoxygenase inhibitor N-benzyl-N-hydroxy-5-phenylpentanamide inhibited 12-HETE production and the ability to adhere to endothelial cells and the formation of new tumours in the lung (Liu *et al.*, 1994).

The exogenous addition of 12-HETE induces time- and dose-dependent endothelial cell retraction in both large and micro-vessels (Honn *et al.*, 1994a, 1994b).

12-HETE regulates the expression of receptor-mediated adhesion of tumour cells to endothelial cells, sub-endothelial matrix and fibronectin (Honn *et al.*, 1988).

Pretreatment of murine melanoma tumour cells with exogenous 12-HETE enhances αIIbβ-integrin mediated adhesion to and spreading on fibronectin (Timar, *et al.*, 1992).

In rat prostate adenocarcinoma, 12 HETE increases the motility and invasion of tumour cells (Lui *et al.*, 1997).

Recent studies (Steinber, 1999; Cyrus *et al.*, 1999) have demonstrated the role of lipoxygenases, particularly 12/15-lipoxygenases, in the pathogenesis of atherosclerosis, and suggest that inhibition of these lipoxygenases may decrease disease progression. Atherosclerosis is regarded as the underlying cause of myocardial infarction, stroke and vascular occlusive disease of the extremities, and is the leading cause of mortality in countries such as the United States of America. Accordingly, inhibitors of lipoxygenases have a role in the prevention and/or treatment of atherosclerosis.

SUMMARY OF THE INVENTION

International Patent Application No. PCT/AU96/00564 discloses a preparation having anti-inflammatory activity, particularly anti-arthritic activity, which comprises a lipid extract of *Perna canaliculus* or *Mytilus edulis* rich in non-polar lipids, which is prepared by supercritical fluid extraction from crude mussel powder.

In work leading to the present invention, it has been demonstrated that the lipid extract disclosed in International Patent Application No. PCT/AU96/00564 is an effective inhibitor of LTB_4 and 5-HETE synthesis in isolated human polymorphonuclear neutrophils and of 12-HETE production by human platelets.

Accordingly, in one aspect the present invention provides a method of inhibition of a lipoxygenase pathway, particularly the 5-lipoxygenase pathway

and/or the 12-lipoxygenase pathway, which comprises administration of an effective amount of a lipid extract of *Perna canaliculus* or *Mytulis edulis*.

In another aspect, the present invention provides a method for inhibition of leukotriene synthesis, particularly inhibition of LTB_4, LTC_4, LTD_4 and LTE_4 synthesis, which comprises administration of an effective amount of a lipid extract of *Perna canaliculus* or *Mytulis edulis*.

In a further aspect, the present invention provides a method for the treatment of a disease or condition associated with a lipoxygenase pathway, particularly the 5-lipoxygenase pathway and/or the 12-lipoxygenase pathway, in a human or animal patient which comprises administration to the patient of an effective amount of a lipid extract of *Perna canaliculus* or *Mytulis edulis*.

Preferably, the lipid extract is an extract rich in non-polar lipids as described in International Patent Application No. PCT/AU96/00564, particularly a lipid extract prepared by supercritical fluid extraction from crude mussel powder.

The present invention extends to the use of a lipid extract of *Perna canaliculus* or *Mytulis edulis* in the preparation of a composition for use in inhibition of a lipoxygenase pathway, particularly in inhibition of the 5-lipoxygenase pathway and/or the 12-lipoxygenase pathway.

The invention also extends to the use of a lipid extract of *Perna canaliculus* or *Mytulis edulis* in the preparation of a composition for use in inhibition of leukotriene synthesis, particularly inhibition of LTB_4, LTC_4, LTD_4 and LTE_4 synthesis.

In yet another aspect, this invention extends to the use of a lipid extract of *Perna canaliculus* or *Mytilus edulis* in the preparation of a composition for use in

treatment of a disease or condition associated with a lipoxygenase pathway, particularly the 5-lipoxygenase pathway and/or the 12-lipoxygenase pathway, in a human or animal patient.

The present invention also extends to a composition for inhibition of a lipoxygenase pathway, particularly the 5-lipoxygenase pathway and/or the 12-lipoxygenase pathway, which comprises a lipid extract of *Perna canaliculus* or *Mytilus edulis*, together with one or more pharmaceutically acceptable carriers and/or diluents.

As used herein, the term "treatment" extends to both prophylactic and therapeutic treatment of the particular disease or condition in the patient.

As used herein, the term "disease or condition associated with a lipoxygenase pathway" is used to encompass all diseases or conditions in which metabolites of a lipoxygenase pathway (particularly the 5-lipoxygenase pathway and/or the 12-lipoxygenase pathway) play a role, and in which at least partial inhibition of the lipoxygenase pathway can provide an effective treatment. These diseases or conditions include, by way of example:

- respiratory diseases or conditions such as asthma, bronchial disease and chronic obstructive pulmonary disease (COPD);
- vascular diseases or conditions such as atherosclerosis, coronary artery diseases, hypertension and sickle cell disease-associated vaso-occlusion;
- skin diseases or conditions such as various dermatitis, psoriasis and atopic eczema;
- gastrointestinal diseases or conditions such as inflammatory bowel disease, ulcerative colitis, Crohn's disease, pancreatitis and periodontal disease;
- cancers such as bowel cancer and prostate cancer;
- sarcoidosis;
- septic shock;

- musculo-skeletal diseases or conditions such as arthritis, including polyarthritis and rheumatoid arthritis;
- leukemia;
- diabetes;
- allergy including otitis media and ocular allergy;
- uveitis;
- dysmenorrhoea;
- kidney diseases or conditions such as glomerulonephritis and nephrotic syndrome; and
- prostate diseases or conditions such as benign prostate hyperplasia.

Throughout this specification, unless the context requires otherwise, the word "comprise", and or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

DETAILED DESCRIPTION OF THE INVENTION

As described above, work leading to the present invention has demonstrated that the lipid extract described herein is an effective inhibitor of the 5-lipoxygenase and 12-lipoxygenase pathways, inhibiting production of metabolites of these pathways including LTB_4, LTC_4, LTD_4 and LTE_4, 5-HETE and 12-HETE. Since LTB_4, LTC_4, LTD_4 and LTE_4 production is massively increased during an asthma attack, inhibition of production of these leukotrienes is a mechanism of action whereby the lipid extract may be effective in the treatment of asthma. Similarly, since it has been demonstrated that 5-HETE is involved in the proliferative response of cancer cells, and that 12-HETE is involved in tumour metastasis, inhibition of 5-HETE and/or 12-HETE synthesis is a mechanism of action whereby the lipid extract may be effective in the treatment of cancer.

Preferably, the lipid extract which is used in the methods of the present invention is an extract prepared by supercritical fluid extraction (SFE) of freeze-dried powdered mussel using a cryogenic fluid (such as cryogenic fluid CO_2) as the extracting medium. This method of preparation is fully described in International Patent Application No. PCT/AU96/00564, the contents of which are incorporated herein by reference. In comparison to solvent extraction techniques, supercritical fluid extraction using cryogenic fluid CO_2 produces a lipid extract rich in non-polar lipids, particularly in free fatty acids. While the exact composition of the lipid extract has not yet been established, it is known to contain not only free fatty acids (including unsaturated fatty acids), but also triglycerides and cholesterol esters.

A variety of administration routes are available. The particular mode selected will depend, of course, upon the particular condition being treated and the dosage required for therapeutic efficacy. The methods of this invention, generally speaking, may be practised using any mode of administration that is medically acceptable, meaning any mode that produces therapeutic levels of the active component of the invention without causing clinically unacceptable adverse effects. Such modes of administration include oral, rectal, topical, nasal, transdermal or parenteral (e.g. subcutaneous, intramuscular and intravenous) routes.

Compositions comprising the lipid extract may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. Such methods include the step of bringing the active component into association with a carrier which constitutes one or more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing the active component into association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product.

Compositions suitable for oral administration may be presented as discrete units such as capsules, cachets, tablets or lozenges, each containing a

predetermined amount of the active component, in liposomes or as a suspension in an aqueous liquid or non-liquid such as a syrup, an elixir, or an emulsion.

Compositions suitable for parenteral administration conveniently comprise a sterile aqueous preparation of the active component which is preferably isotonic with the blood of the recipient. This aqueous preparation may be formulated according to known methods using those suitable dispersing or wetting agents and suspending agents. A sterile injectable preparation may be formulated as a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent, for example as a solution in polyethylene glycol and lactic acid. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose, any bland fixed oil may be employed including synthetic mono-or di-glycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables.

Other delivery systems can include sustained release delivery systems. Preferred sustained release delivery systems are those which can provide for release of the active component of the invention in sustained release pellets or capsules. Many types of sustained release delivery systems are available. These include, but are not limited to: (a) erosional systems in which the active component is contain within a matrix, and (b) diffusional systems in which the active component permeates at a controlled rate through a polymer.

The formulation of such therapeutic compositions is well known to persons skilled in this field. Suitable pharmaceutically acceptable carriers and/or diluents include any and all conventional solvents, dispersion media, fillers, solid carriers, aqueous solutions, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like. The use of such media and agents for pharmaceutically active substances is well known in the art, and it is described, by

way of example in *Remington's Pharmaceutical Sciences*, 18th Edition, Mack Publishing Company, Pennsylvania, USA. Except insofar as any conventional media or agent is incompatible with the active component, use thereof in the pharmaceutical compositions of the present invention is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

Oral administration will be preferred for many conditions because of the convenience to the patient, although parenteral administration or localised sustained delivery may be more desirable for certain treatment regimens.

The active component is administered in therapeutically effective amounts. A therapeutically effective amount means that amount necessary at least partly to attain the desired effect, or to delay the onset of, inhibit the progression of, or halt altogether, the onset or progression of the particular condition being treated. Such amounts will depend, of course, on the particular condition being treated, the severity of the conditions and individual patient parameters including age, physical condition, size, weight and concurrent treatment. These factors are well known to those of ordinary skill in the art and can be addressed with no more than routine experimentation. It is preferred generally that a maximum dose be used, that is, the highest safe dose according to sound medical judgement. It will be understood by those of ordinary skill in the art, however, that a lower dose or tolerable dose may be administered for medical reasons, psychological reasons or for virtually any other reasons.

It is especially advantageous to formulate compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the human or animal patients to be treated; each unit containing a predetermined quantity of active component calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier and/or diluent. The

specifications for the novel dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active component and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active component for the particular treatment.

Generally, daily doses of active component will be from about 0.01 mg/kg per day to 1000 mg/kg per day. Small doses (0.01-1 mg) may be administered initially, followed by increasing doses up to about 1000 mg/kg per day. In the event that the response in a subject is insufficient at such doses, even higher doses (or effective higher doses by a different, more localised delivery route) may be employed to the extent patient tolerance permits. Multiple doses per day are contemplated to achieve appropriate systemic levels of the active component.

In the accompanying figures:

Figure 1 shows the metabolism of arachidonic acid via the lipoxygenase pathways.

Figure 2 shows the effect of lipid extract on platelet 12-HETE and neutrophil 5-HETE synthesis.

Further features of the present invention are more fully described in the following Example(s). It is to be understood, however, that this detailed description is included solely for the purposes of exemplifying the present invention, and should not be understood in any way as a restriction on the broad description of the invention as set out above.

EXAMPLE 1

A PREPARATION OF LIPID EXTRACT

A.1 Raw Material

The green lipped mussel (*Perna canaliculus*) is harvested on the south coast of New Zealand at which time the total mussel is stabilised with tartaric acid. Freeze drying results in a dry power of pulverised form.

A.2 Extraction of Lipids

The technique of supercritical fluid extraction (SFE) is utilised to extract the biologically active lipids from the crude mussel powder. Cryogenic fluid CO_2 is used as the extracting medium. The CO_2 is expanded to atmospheric pressure and the extract is presented as a concentrated oil. The powder yields 3-3.5% of oil.

A.3 Profile of the crude oil

The extractable oil is orange amber in colour and is a viscous liquid at ambient temperature. The extract is stored below 4°C and is handled in a nitrogen atmosphere. The crude oil shows strong UV activity and is protected from light to minimise the polymerisation of double bond components.

B PILOT SCALE SUPERCRITICAL FLUID EXTRACTION

Extraction of total lipids in freeze-dried mussel powder, *Perna canaliculus* was performed on a pilot scale SFE unit undertaken at the Food Research Institute (Department of Agriculture, Werribee, Vic., Australia).

B.1 Instrumentation

Extractions were performed on a pilot scale extraction unit consisting of five basic sub-units (Distillers MG Limited., England, UK). The five basic units comprise: Carbon dioxide supply, Solids extraction, Primary separation, Evaporation and Tailing units.

The carbon dioxide supply unit consists of two CO_2 cylinders connected in parallel and placed on a weighing scale for recharging when appropriate. The extraction unit can be supplied with liquid SC-CO_2 and SC-CO_2. For this work the SFE unit was operated using SC-CO_2. Solid material was placed in the leaching column and the primary separator facilitates separation of extracted material by reduction of pressure (which allows extract to settle), adsorption or liquid extraction. The fluid extract was passed into the evaporation unit to evaporate the CO_2 by the use of internal heating tubes. The vapour may contain volatiles and thus it is subsequently passed to the tailing column to be scrubbed by pure liquid CO_2. The tailing unit traps the gaseous CO_2 from the evaporator unit and returns the volatile components to the evaporator.

B.2 Pilot plant extraction procedure

Mussel power (300 g) was charged to the extraction unit (leaching column). SC-CO_2 was delivered at a flow rate of 3.0 kg/h for two hours per extraction. Extractor temperature was set at 40°C and the extractor pressure at 310 bar (4,500 psi). The evaporator temperature was held constant at 40°C. The mussel lipid extracts were stored under nitrogen at -10°C in amber glass sealed containers.

EXAMPLE 2

Recent research (see above) has demonstrated that 5-HETE is involved in the proliferative response of cancer cells. In addition, it has also been demonstrated that the product of the 12-lipoxygenase pathway, 12-HETE, is involved in tumour metastasis (see above). As a result of this overwhelming evidence, studies were undertaken to examine the inhibitory effect of the lipid extract prepared by SFE as described in Example 1 on the production of 12-HETE by platelets, and to compare it with its inhibitory effects on 5-HETE synthesis by neutrophils.

To do this, isolated human platelets or neutrophils were pre-incubated with different doses of the lipid extract for 10 minutes, before 12-HETE synthesis was initiated by the addition of 10 µM arachidonic acid and 5 µM A23187 (calcium ionophore). Synthesis was allowed to proceed for 5 minutes before it was terminated by the addition of citric acid. Figure 2 shows the effect of increasing concentrations of the lipid extract on both platelet 12-HETE and neutrophil 5-HETE synthesis. 50% inhibition of 12-HETE and 5-HETE was achieved with approximately 10 µg/ml and 30 µg/ml lipid extract respectively.

EXAMPLE 3

In a further series of experiments, the effect of the lipid extract of Example 1 was tested on the production of 5-HETE, LTB_4 and all-trans isomers of LTB_4 in human neutrophils. As can be seen in Table 1, 50 µg/ml lipid extract inhibited LTB_4 synthesis by 62%, the all-trans isomers by 77 and 87% respectively, and 5-HETE synthesis by 88%.

TABLE 1

A. Effect of Lipid Extract on Leukotriene/HETE Synthesis (Expressed as ng product (Mean ± SD) per 10^6 PMN)					
Compound	*Concentration/ Dilution*	*Isomer 1*	*Isomer 2*	*LTB_4*	*5-HETE*
H_2O Control		7.11 ± 1.1	12.4 ± 1.1	10.2 ± 1.3	78 ± 6
Methanol Control		7.1 ± 3.7	11.9 ± 3.5	9.7 ± 1.5	98 ± 8
Lipid Extract (MeOH)	50 µg/ml	1.6 ± 0.4	1.6 ± 1.1	3.7 ± 0.7	12 ± 12
B. Effect of Lipid Extract on Leukotriene/HETE Synthesis (Expressed as a percentage of the Methanol control)					
Compound	*Concentration/ Dilution*	*Isomer 1*	*Isomer 2*	*LTB_4*	*5-HETE*
H_2O Control		100 ± 16	100 ± 9	100 ± 13	100 ± 8
Methanol Control		100 ± 52	100 ± 29	100 ± 15	100 ± 8
Lipid Extract (MeOH)	50 µg/ml	23 ± 6	13 ± 9	38 ± 7	12 ± 12

EXAMPLE 4

The aim of this study was to assess efficacy and safety of a lipid extract prepared by SFE as described in Example 2 in treatment of patients with bronchial asthma. The lipid extract was encapsulated with olive oil as carrier.

Forty patients (14 males and 26 females, aged 18-62 years, median age 40 years) with atopic steroid-naive asthma were enrolled in double-blind randomized placebo control study at the Hospital Therapeutic Clinic of Pavlov's Medical University, St Petersburg, Russia. Thirty patients were treated with the lipid extract (2 capsules, twice daily) for 8 weeks and 10 patients were treated with placebo (olive oil capsules). Inhalations of β_2-antagonists (salbutamol, fenoterol) were used by each group on demand. Patients were diagnosed according to the American Thoracic Society's definition of asthma. Diagnosis was based upon clinical history, reversibility of FEV_1 more than 15%. The patients' mean of duration asthma was

5,8±0,9 years (mean±sem) and their mean FEV_1 at the time of the study was 86,3±3,3% predicted (mean±sem). The study was approved by the Local Ethics Committee. The informed consent of the participants was obtained in writing.

Pulmonary function tests included airway resistance, specific airway conductance ("Respiratory system 3000", Ohio Medical Products, Madison, USA), forced vital capacity, FEV_1, mid-expiratory flow at 25, 50 and 75% of vital capacity ("Pneumoscreen II", Jaeger, Hoechberg, Germany). For assessment of peak flow rate, individual peak-flow meters were used (Vitalograph for Allersearch, Ireland). The concentrations of eosinophil cationic protein (ECP) were determined using radioimmunoassay (Pharmacia & Upjohn, Uppsala, Sweden). The concentration of hydrogen peroxide in exhaled air condensates was measured using horse radish peroxidase-catalysed oxidation of tetramethylbenzidine. Students paired two-tailed t-test was used for statistical methods (Microsoft Excel 5, Statistica for Windows 5). P value less than 0.05 was considered significant.

The results of the study are shown in Tables 2 and 3. The lipid extract had a positive effect on clinical symptoms, peak expiratory flow (PEF) rate and concentration of hydrogen peroxide in exhaled air condensate. There was no improvement in the placebo treated group. No side effects were observed in either group of asthmatic patients during the treatment with the lipid extract or placebo. Table 4 provides a summary analysis of these results.

In conclusion, this study has revealed beneficial effects of the lipid extract in mild asthmatic patients.

TABLE 2

Efficacy of Lipid Extract in Patients with Bronchial Asthma			
	Baseline	*28 Days*	*56 Days*
Chest Tightness (times/day)	1,57 ± 0,29	0,89 ± 0,18*	1,0 ± 0,42*
Night Awakeness (times/day)	0,54 ± 0,12	0,25 ± 0,10*	0,16 ± 0,09*
Usage of β_2-antagonists (puffs/day)	1,61 ± 0,44	0,77 ± 0,30*	0,87 ± 0,35*
FEV_1 (% predicted)	80,29 ± 3,91	71,96 ± 3,77	81,29 ± 3,86
PEF (l/min)	360,35 ± 15,52	366,07 ± 18,51	398,75 ± 18,32*
Hydrogen Peroxide in Breath Air Condensate (µM)	0,135 ± 0,025	0,086 ± 0,014*	0,058 ± 0,009*
ECP in Serum (µg/l)	6,57 ± 1,22	8,03 ± 2,26	5,77 ± 0,87 (n=17)

Values are present as mean ± sem. *$p < 0.05$ versus baseline

TABLE 3

Clinical Symptoms, Lung Function Tests and Markers of Airway Inflammation in Asthmatic Patients Treated with Placebo			
	Baseline	*28 Days*	*56 Days*
Chest Tightness (times/day)	1,0 ± 0,30	0,81 ± 0,32	0,87 ± 0,19
Night Awakeness (times/day)	0,1 ± 0,08	0,16 ± 0,11	0,18 ± 0,12
Usage of β_2-antagonists (puffs/day)	0,08 ± 0,08	0,16 ± 0,16	0,27 ± 0,27
FEV_1 (% predicted)	100,18 ± 3,6	83,6 ± 3,0	101,49 ± 2,88
PEF (l/min)	461,66 ± 26,82	447,27 ± 40,23	465,45 ± 37,84
Hydrogen Peroxide in Breath Air Condensate (µM)	0,21 ± 0,12	0,14 ± 0,50	0,09 ± 0,20
ECP in Serum (µg/l)	6,19 ± 1,02	4,82 ± 0,92	7,42 ± 1,84

Values are present as mean ± sem.

TABLE 4

Summary Analysis of Results			
	Lipid Extract	*Placebo*	*%Efficacy*
Chest Tightness	↓ 40%	↓ 13%	27%
Night Awakening	↓ 70%	↑ 80%	150%
Usage of β_2-antagonists (puffs/day)	↓ 50%	↑ 300%	350%
Peak Expiration Flow	↑ 15%	-	15%

REFERENCES:

Anderson KM, Seed T, Meng J, Ou D, Alrefai WA and Harris JE (1998). Five-lipoxygenase inhibitors reduce Panc-1 survival: the mode of cell death and synergism of MK886 with gamma linolenic acid. *Anticancer Res* **18**: 791-800.

Cyrus T, Witztum JL, Rader DJ, Tangirala R, Fazio S, Linton MF and Funk CD (1999). Disruption of the 12/15-lipoxygenase gene diminishes atherosclerosis in apo E-deficient mice. *J Clin Invest* **103**: 1597-1604.

Gao X, Grignon DJ, Chbihi T, Zacharek A, Chen YQ, Sakr W, Porter AT, Crissman JD, Pontes JE, Powell IJ and et al. (1995). Elevated 12-lipoxygenase mRNA expression correlates with advanced stage and poor differentiation of human prostate cancer. *Urology* **46**: 227-237.

Ghosh J and Myers CE (1997). Arachidonic acid stimulates prostate cancer cell growth: critical role of 5-lipoxygenase. *Biochem Biophys Res Commun* **235**: 418-423.

Hofmanova J, Musilova E and Kozubik A (1996). Suppression of human cancer cell proliferation by lipoxygenase inhibitors and gamma-radiation in vitro. *Gen Physiol Biophys* **15**: 317-331.

Honn KV, Grossi IM, Fitzgerald LA, Umbarger LA, Diglio CA and Taylor JD (1988). Lipoxygenase products regulate IRGpIIb/IIIa receptor mediated adhesion of tumor cells to endothelial cells, subendothelial matrix and fibronectin. *Proc Soc Exp Biol Med* **189**: 130-135.

Honn KV, Grossi IM, Steinert BW, Chopra H, Onoda J, Nelson KK and Taylor JD (1989). Lipoxygenase regulation of membrane expression of tumor cell

glycoproteins and subsequent metastasis. *Adv Prostaglandin Thromboxane Leukot Res* **19**: 439-443.

Honn KV, Tang DG, Grossi I, Duniec ZM, Timar J, Renaud C, Leithauser M, Blair I, Johnson CR, Diglio CA and et al. (1994a). Tumor cell-derived 12(S)-hydroxyeicosatetraenoic acid induces microvascular endothelial cell retraction. *Cancer Res* **54**: 565-574.

Honn KV, Tang DG, Grossi IM, Renaud C, Duniec ZM, Johnson CR and Diglio CA (1994b). Enhanced endothelial cell retraction mediated by 12(S)-HETE: a proposed mechanism for the role of platelets in tumor cell metastasis. *Exp Cell Res* **210**: 1-9.

Hussey HJ and Tisdale MJ (1996). Inhibition of tumour growth by lipoxygenase inhibitors. *Br J Cancer* **74**: 683-687.

Ichikawa T, Uchida M, Murakami A, Yano T, Yano Y and Otani S (1997). The inhibitory effect of vitamin E on arachidonic acid metabolism during the process of urethane-induced lung tumorigenesis in mice. *J Nutr Sci Vitaminol* (Tokyo) **43**: 471-477.

Liu B, Marnett LJ, Chaudhary A, Ji C, Blair IA, Johnson CR, Diglio CA and Honn KV (1994). Biosynthesis of 12(S)-hydroxyeicosatetraenoic acid by B16 amelanotic melanoma cells is a determinant of their metastatic potential. *Lab Invest* **70**: 314-323.

Liu B, Maher RJ, Jonckheere JP, Popat RU, Stojakovic S, Hannun YA, Porter AT and Honn KV (1997). 12(S)-HETE increases the motility of prostate tumor cells through selective activation of PKC alpha [In Process Citation]. *Adv Exp Med Biol* 707-718.

Natarajan R, Esworthy R, Bai W, Gu JL, Wilczynski S and Nadler J (1997). Increased 12-lipoxygenase expression in breast cancer tissues and cells. Regulation by epidermal growth factor. *J Clin Endocrinol Metab* **82**: 1790-1798.

Steinberg D (1999). At last, direct evidence that lipoxygenases play a role in atherogenesis. *J Clin Invest* **103**: 1487-1488

Tavares IA, Stamford IF and Bennett A (1987). Tamoxifen inhibits 5-lipoxygenase in human polymorphonuclear leucocytes. *J Pharm Pharmacol* **39**: 323-324.

Teicher BA, Korbut TT, Menon K, Holden SA and Ara G (1994). Cyclooxygenase and lipoxygenase inhibitors as modulators of cancer therapies. *Cancer Chemother Pharmacol* **33**: 515-522.

Timar J, Chen YQ, Liu B, Bazaz R, Taylor JD and Honn KV (1992). The lipoxygenase metabolite 12(S)-HETE promote αIibβ3 integrin mediated tumor cell spreading on fibronectin. *Int J Cancer* **52**: 594-603.

CLAIMS

1. A method of inhibition of a lipoxygenase pathway, particularly the 5-lipoxygenase pathway and/or the 12-lipoxygenase pathway, which comprises administration of an effective amount of a lipid extract of *Perna canaliculus or Mytulis edulis.*

2. A method for inhibition of leukotriene synthesis, particularly inhibition of LTB_4, LTC_4, LTD_4 and LTE_4 synthesis, which comprises administration of an effective amount of a lipid extract of *Perna canaliculus or Mytulis edulis.*

3. A method for the treatment of a disease or condition associated with a lipoxygenase pathway, particularly the 5-lipoxygenase pathway and/or the 12-lipoxygenase pathway, in a human or animal patient which comprises administration to the patient of an effective amount of a lipid extract of *Perna canaliculus or Mytulis edulis.*

4. A method according to any one of claims 1 to 3, wherein the lipid extract is an extract rich in non-polar lipids.

5. A method according to claim 4, wherein the lipid extract is prepared by supercritical fluid extraction from crude mussel powder.

6. A method according to claim 3, wherein the treatment is treatment of cancer, particularly inhibition of tumour cell proliferation or inhibition of tumour metastasis.

7. A method according to claim 3, wherein the treatment is treatment of asthma.

8. A method according to claim 3, wherein the treatment is treatment of atherosclerosis.

9. Use of a lipid extract of *Perna canaliculus or Mytulis edulis*, in the preparation of a composition for use in inhibition of a lipoxygenase pathway, particularly in inhibition of the 5-lipoxygenase pathway and/or the 12-lipoxygenase pathway.

10. Use of a lipid extract of *Perna canaliculus or Mytulis edulis*, in the preparation of a composition for use in inhibition of leukotriene synthesis, particularly inhibition of LTB_4, LTC_4, LTD_4 and LTE_4 synthesis.

11. Use of a lipid extract of *Perna canaliculus or Mytulis edulis*, in the preparation of a composition for use in treatment of a disease or condition associated with a lipoxygenase pathway, particularly the 5-lipoxygenase pathway and/or the 12-lipoxygenase pathway, in a human or animal patient.

12. A composition for inhibition of a lipoxygenase pathway, particularly the 5-lipoxygenase pathway and/or the 12-lipoxygenase pathway, which comprises a lipid extract of *Perna canaliculus or Mytulis edulis*, together with one or more pharmaceutically acceptable carriers and/or diluents.

AMENDED CLAIMS

[received by the International Bureau on 3 July 2000 (03.07.00);
original claims 1-12 replaced by new claims 1-13 (2 pages)]

1 A method of inhibition of the synthesis of 5-HETE and/or 12-HETE, which comprises administration of an effective amount of a lipid extract of *Perna canaliculus or Mytulis edulis*.

2 A method for the treatment of cancer in a human or animal patient, which comprises administration to the patient of an effective amount of a lipid extract of *Perna canaliculus or Mytulis edulis*.

3. A method according to claim 1 or claim 2, wherein the lipid extract is an extract rich in non-polar lipids.

4. A method according to claim 3, wherein the lipid extract is an extract prepared by supercritical fluid extraction

5. A method according to claim 4, wherein the lipid extract is an extract prepared by supercritical fluid extraction from crude mussel powder.

6. A method according to claim 2, wherein the treatment is inhibition of tumour cell proliferation or inhibition of tumour metastasis.

3 Use of a lipid extract of *Perna canaliculus or Mytulis edulis*, in the preparation of a composition for use in inhibition of the synthesis of 5-HETE and/or 12-HETE.

8. Use of a lipid extract of *Perna canaliculus or Mytulis edulis*, in the preparation of a composition for use in treatment of cancer in a human or animal patient.

9. Use according to claim 7 or claim 8, wherein the lipid extract is an extract rich in non-polar lipids.

10. Use according to claim 9, wherein the lipid extract is an extract prepared by supercritical fluid extraction.

11. Use according to claim 10, wherein the lipid extract is an extract prepared by supercritical fluid extraction from crude mussel powder.

12. A composition for use in inhibition of the synthesis of 5-HETE and/or 12-HETE, which comprises a lipid extract of *Perna canaliculus or Mytulis edulis*, together with one or more pharmaceutically acceptable carriers and/or diluents.

13. A composition for use in the treatment of cancer in a human or animal patient, which comprises a lipid extract of *Perna canaliculus* or *Mytulis edulis*, together with one or more pharmaceutically acceptable carriers and/or diluents.

Figure 1

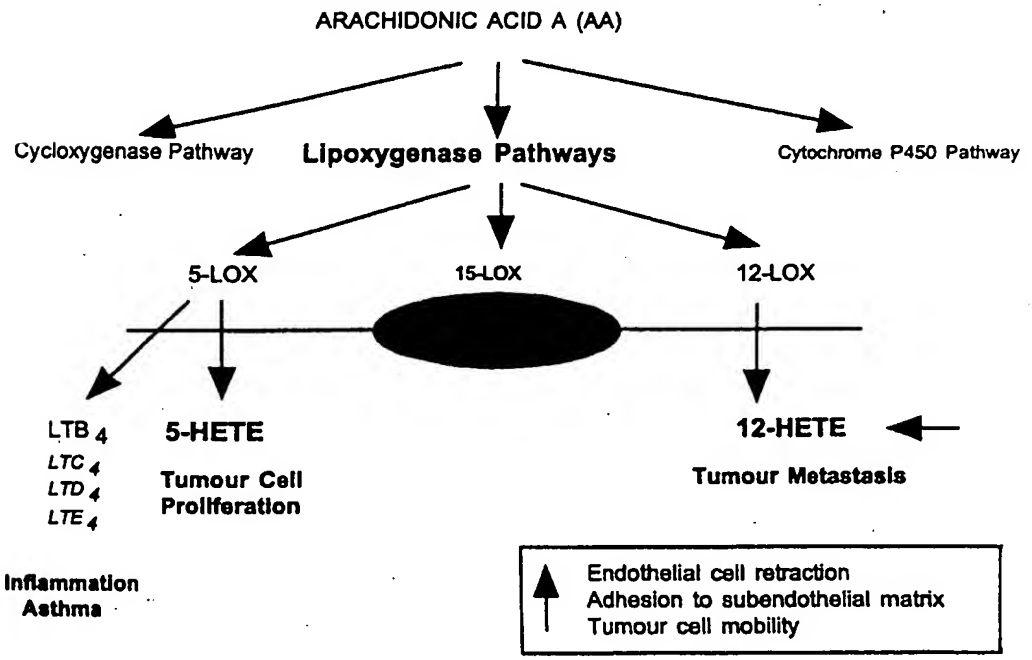
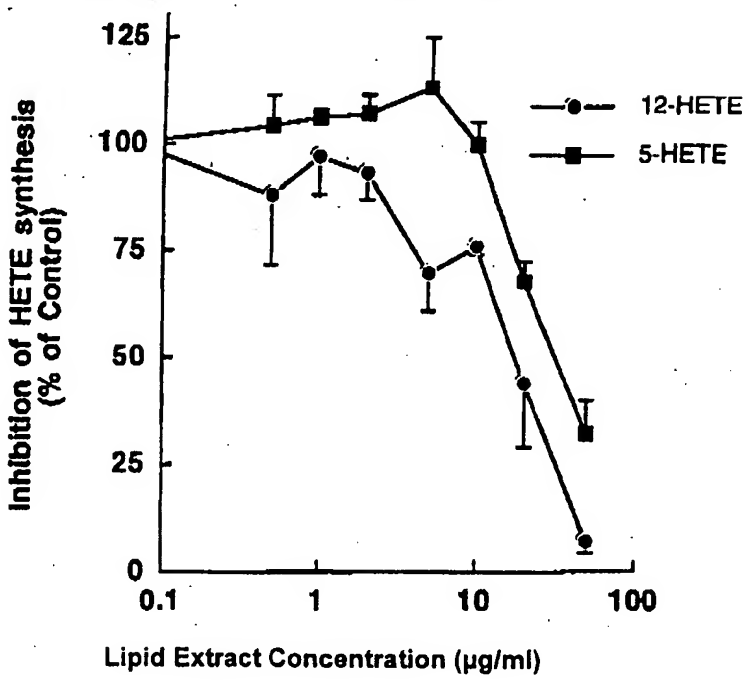

ARACHIDONIC ACID A (AA)
Cycloxygenase Pathway
Lipoxygenase Pathways
Cytochrome P450 Pathway
5-LOX
15-LOX
12-LOX
LTB 4
LTC 4
LTD 4
LTE 4
5-HETE
Tumour Cell Proliferation
12-HETE
Tumour Metastasis
Inflammation
Asthma
Endothelial cell retraction
Adhesion to subendothelial matrix
Tumour cell mobility

Figure 2

Effect of Lipid Extract on 12-HETE and 5-HETE synthesis by human platelets and neutrophils respectively



INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 00/00179

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[7]: A61K 35/56; A61P 35/00, 35/04, 11/06, 9/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K and keywords as set out below

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
AU: IPC as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
DERWENT : PERNA() CANALICULUS OR MYTULIS() EDULIS OR GREEN() LIPPED() MUSSEL OR
JAPIO : BLUE() MUSSEL
CA :
MEDLINE :

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Inflammopharmacology, volume 5, 1997, Kluwer Academic Publishers, Netherlands, Whitehouse, M.W. et al, "Anti-inflammatory activity of a lipid fraction (Lyprinol) from the New Zealand green-lipped mussel", pages 237-246 abstract, pages 244-245	1-12
X	Arzneimittel Forschung/Drug Research, volume 30, No. (II), No. 12, 1980, Editio Cantor, Aulendorf, Germany, K.D. Rainsford et al, "Gastroprotective and Anti-inflammatory Properties of Green Lipped Mussel (Pera canaliculus) Preparation", pages 2128-2132 page 2130	12

[X] Further documents are listed in the continuation of Box C

[X] See patent family annex

* Special categories of cited documents:

"A" Document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
07 April 2000	- 2 MAY 2000
Name and mailing address of the ISA/AU	Authorized officer
AUSTRALIAN PATENT OFFICE PO BOX 200 WODEN ACT 2606 AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No.: (02) 6285 3929	STEVEN CHEW Telephone No.: (02) 6283 2248

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 00/00179

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96/05164 A (J. W. BROADBENT NOMINEES PTY LTD) 22 February 1996 page 5, line 23-page 6, line 3; claims 17-19	1-5,7,9-12
X	WO 97/09992 A (J. W. BROADBENT NOMINEES PTY LTD) 20 March 1997 page 2, line 23-page 3, line 13	12

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/AU 00/00179

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
WO	05164/96	AU	31565/95	CA	2196422	EP	777641
		ZA	9506661				
WO	09992/97	AU	68656/96	CA	2231803	EP	850068

END OF ANNEX